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RECOMBINANT IMMUNOCASTRATION VACCINE

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(57) Claim

1. A recombinant immunocastration vaccine suitable for use in an animal and capable of stimulating the production of antibodies to LHRH in said animal, said vaccine comprising a polypeptide containing an LHRH amino acid sequence or an analogue thereof, one or more T-cell epitopes and a purification site.

14. A method for immunocastrating an animal, said method comprising administering to said animal an antibody-stimulating effective amount of a polypeptide, said polypeptide comprising an LHRH amino acid sequence or an analogue thereof, one or more T-cell epitopes and a purification site, for a time and under conditions sufficient for said animal to generate LHRH specific antibodies.

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Complete Specification for the invention entitled:

"Recombinant Immunocastration Vaccine"

The following statement is a full description of this invention, including the best method of performing it known to us:

RECOMBINANT IMMUNOCASTRATION VACCINE

The present invention relates generally to an immunocastration vaccine and more particularly is directed to a vaccine composition comprising luteinizing hormone releasing hormone or an analogue thereof. The present invention further contemplates a method for the immunological castration of animals.

An alternative to surgical castration is active immunisation against luteinizing hormone releasing hormone (hereinafter referred to as "LHRH") or an analogue thereof. LHRH is released from the hypothalamus into the pituitary portal vessels. It has been suggested that LHRH binds to the gonadotrophs of the anterior pituitary to stimulate secretion and release of luteinizing hormone (LH). These hormones act on the sex organs, ovary and uterus in females or testis in males to

produce both sex hormones (either oestrogens or androgens) and also to allow cellular differentiation within sex organs leading to production of active gamets. Active immunisation against LHRH is proposed to disrupt the communication between the hypothalamus and pituitary. This active immunisation may lead to complete inhibition of sexual function in both males and females. It is thought that the site at which antibodies to LHRH might inhibit reproductive function is in the hypophyseal portal blood vessels.

Although active immunisation against LHRH has been achieved in some animals, individual animals vary significantly in their response to immunisation. This is probably due in part to the size of the LHRH molecule and how it is presented to an animal's immune system.

In current vaccines, the naturally-occurring decapeptide of LHRH[1] or various modifications or truncations thereof[2] is chemically coupled to a suitable carrier protein which acts as a source of T-cell epitopes. However, there are a number of problems associated with this approach which are summarised as follows:

1. **Selection of carrier protein:**

A number of proteins are used as peptide carriers. These include diptheria toxoid (DT), tetanus toxoid (TT), keyhole limpet haemocyanin, ovalbumin, bovine serum albumin and the like. The most successful proteins have been DT and TT, presumably because they contain powerful T-cell epitopes with broad species reactivities. All of these proteins are, however, reasonably expensive to produce at the level of purity required for vaccine production. Additionally, the toxins of DT and TT require chemical detoxification prior to use which adds a further cost and uses surface reactive groups which would

otherwise be available for peptide conjugation.

2. Source of LHRH:

The naturally occurring decapeptide LHRH or one of various analogues thereof (e.g. the nonapeptide 2-10) can be used for coupling to the carrier. Peptide can be manufactured in commercial quantities by chemical or enzymic synthesis. However, both processes are subject to error in synthesis and significant levels of impurity can survive extensive purification procedures. The final cost of purified peptide, even in commercial quantities, is expensive and becomes a major component of vaccine costs.

3. Chemical coupling of LHRH to carrier protein:

There are various well documented chemical procedures whereby LHRH can be coupled to a carrier protein. The choice of procedure will be determined by availability of reactive groups on the carrier protein, available reactive groups on LHRH or the chosen analogue, and acceptability of chemicals to vaccine registration authorities and the efficiency of the coupling procedure. Regardless of the choice of carrier protein, peptide and coupling reagent, coupling efficiency is rarely better than 40% to 50% and formation of aggregates are difficult to avoid.

There is a need, therefore, to develop an LHRH based vaccine which is highly pure, chemically fully definable, reproducible, cost effective in production and efficacious.

Accordingly, one aspect of the present invention contemplates a recombinant immunocastration vaccine suitable for use in an animal and capable of stimulating the production of antibodies to LHRH in said animal, said vaccine comprising a polypeptide containing an LHRH amino

acid sequence or an analogue thereof, one or more T-cell epitopes and a purification site.

By "vaccine" as used herein is meant a composition of matter capable of stimulating a humoral and optionally also a cell-mediated immune response and/or any combinations thereof.

The "purification site" may be naturally occurring within the polypeptide molecule such as a glutathione-binding site on glutathione-S-transferase (GST;3) or may be added to the molecule as part of the genetic engineering process. An example of the latter is the addition of a hexa-histidine (4) to a suitable antigen.

The T-cell epitope of the vaccine polypeptide is necessary such that the animal species for which the vaccine is intended can recognise and respond to it. The polypeptide may contain a single epitope or more than one. The choice of the source of T-cell epitopes will vary depending on the animal to be vaccinated.

The LHRH amino acid sequence or its analogues (including its derivatives) may be represented once in the polypeptide or as tandem or multiple repeats within the polypeptide or at its C-terminal end. Derivatives and analogues of LHRH include any single or multple amino acid substitutions, deletions and/or additions to the naturally occurring or recombinant amino acid sequence. Such alterations to the amino acid sequence of LHRH may occur at the amino terminal or carboxy terminal ends of the peptide or may occur within the peptide molecule itself. The analogues and derivatives of LHRH contemplated herein may not necessarily function as a LH stimulating factor but may nevertheless have antibodies raised against it which will cross react with naturally occurring LHRH. Furthermore, the range of analogues and

derivatives encompassed by the present invention also includes chemical alterations to the amino acid residues such as amidation. For example, the present invention extends to LHRH without C-terminal amidation.

Where the LHRH amino acid sequence is represented more than once, it may be as a tandem or multiple repeat, with or without spacers, at intervals throughout the carrier molecule or at intervals throughout the carrier molecule and including within the sequence an enzymic cleavage site so that the expressed molecule can be converted enzymically into a number of peptides each of which will contain T-cell epitopes derived from the carrier molecule and a C-terminal LHRH. The enzymes can be chosen to yield free carboxy or amidated LHRH.

In any event, reference herein to "LHRH" includes all such analogues or derivatives of LHRH, represented singly or multiply in the vaccine polypeptide.

In a preferred embodiment, the recombinant immunocastration vaccine further comprises a capacity for self-assembly such that the polypeptide can self-assemble into virion like-structures (e.g. Hepatitis^5 B core antigen (HepB)) or into filaments (e.g. Potyvirus).

The fusion polypeptide of the present invention therefore provides a carrier molecule (e.g. GST) which is an integral part of the vaccine.

The vaccine of the present invention may be administered parenterally, for example, by subcutaneous, intramuscular and/or intraveneous injection. Administration may also be orally, nasally, or by adsorption through the skin by microsponges or a mini-pump either implanted in the animal or attached outside the animal.

Vaccine formulations may also comprise one or more pharmaceutically acceptable carriers and/or diluents and may also contain an adjuvant. Examples of adjuvants which may be used are aluminium hydroxide, Freund's complete or incomplete adjuvants, DEAE dextran, levamisole, PCG and polyA, polyC or polyU. Particularly preferred adjuvants are materials which do not cause local inflammation. One example of such material is a mineral oil composition that includes bacterial cell wall material such as peptidoglycans or a synthetic derivative of such a cell wall material. Such a synthetic material is known as muramyl dipeptide.

Reference can conveniently be made to Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Easton, Pennsylvania, U.S.A. for various aspects of vaccine composition preparation.

By "animal" in relation to the suitability of a target for the vaccine includes any animal capable of mounting an immune response and which contains LHRH. Preferably, the animal is a mammal. Even more preferably, the animal is a live stock or domestic animal such as dogs, cats, sheep, cattle, horses, goats or pigs. The present invention further extends to humans.

Another aspect of the present invention contemplates a method for the immunocastration of an animal comprising administering to said animal an antibody-stimulating effective amount of a polypeptide, said polypeptide comprising an LHRH amino acid sequence or analogue thereof, one or more T-cell epitopes and a purification site, for a time and under conditions sufficient for said animal to generate LHRH specific antibodies. These antibodies will eventually result in the animal becoming immunocastrated. The latter condition is considered to occur when the level of LH is

reduced to an extent where the level of testosterone or progesterone is substantially lowered. In accordance with this method, the LHRH amino acid sequence, T-cell epitope and purification site have the same meaning as hereinbefore described. The route of administration may be as described above. Furthermore, the recombinant immunocastration vaccine may be expressed in a microorganism which is, or can become part of, the normal flora of the animal. The engineered microorganism would then secrete an effective amount of the recombinant vaccine. The microorganism may also be mutated such that expression of the vaccine can be induced or repressed depending on environmental stimuli such as diet.

The vaccine may be administered once or multiple administrations may be given. For example, following initial vaccination, booster injections or administrations may be given every 4 to 10 weeks or whenever appropriate for the animal concerned. The age of the animal to be vaccinated will vary depending on the animal but may range from 8 to 50 weeks for calves to 8 to 24 weeks for lambs.

The carrier specifically exemplified herein uses GST derived from Schistosoma japonicum. However, other GST molecules can be used as well as other carrier molecules having purification properties. The carriers of the present invention must also assist in the stimulation of an immune response.

The immunocastration vaccine of the present invention, therefore, is cost effective, chemically defined, efficacious, reproducible and highly pure and, therefore, provides a valuable and useful means for castrating animals.

The present invention is further described by

reference to the following non-limiting Figures and Examples.

In the Figures:

Figure 1A is a photographic representation of a Western blot employing sheep anti-LHRH antiserum screening of the recombinant immunocastration vaccine. Samples 1 to 9 were cells from colony No. 16 - 0, 3, 4 and 0 and 3 hours post addition of IPTG; 0, 3 hours after no additions of IPTG; colony No. 1-0 and 3 hours after addition of IPTG. Thus colony No. 1 was positive for LHRH whereas colony No. 16 was not. Colony No. 16 was chosen for further analysis.

Figure 1B is a photographic representation of a Coomassie stained gel corresponding to the Western blot.

Figure 2 is a graphical representation showing the purification of the recombinant vaccine by glutathione column chromatography.
Samples 1 to 7:
1. cell sonicate.
2. clarified supernatant.
3. unadsorbed material from gluathione.
4. blank
5,6 & 7. 10, 5 & 2 µl sample of eluted peak.

EXAMPLE 1

A) Vaccine Construction

The DNA sequence corresponding to LHRH was inserted into the multiple cloning site of the p-GEX1 plasmid vector (5) by the following method:

Two DNA oligomers each of 38 residues were synthesised and annealed to form a double stranded

oligomer which, upon insertion into the multiple cloning site of the p-GEX1 coded for the LHRH amino acid sequence QHWSYGLRPG in frame with the GST sequence. This double stranded DNA oligomer had the following specific characteristics:

(i) It contained single stranded overhanging ends corresponding to a Bam H1 cohesive sequence at the 5' end and a Eco R1 cohesive sequence at the 3' end. This ensured that the DNA oligomer was inserted into Bam H1/Eco R1 cleaved p-GEX1 in the desired orientation.

(ii) The termination codon TGA immediately followed the sequence coding for the LHRH terminal G amino acid, ensuring this amino acid was the C-terminal residue of the GST/LHRH hybrid protein.

The annealed oligomer was ligated into Bam H1/Eco R1 cleaved p-GEX1 by conventional means. The ligation mix was transformed into competent E. coli K12 strain DH5α cells. Single colonies were selected by overnight growth on Luria agar containing 100 µg/ml carbenicillin. All further growth of transformed cells was carried out in luria broth containing 100 µg/ml carbenicillin (LBC). To test for expression of a GST/LHRH hybrid protein, overnight cultures of colony picks grown in LBC at 37°C were diluted 1:10 in fresh LBC and grown for 1 to 2 hours at 37°C. To induce GST production, IPTG was added to 0.1 mM and growth allowed to proceed for a further 3 to 4 hours at 37°C. Cells were pelleted, lysed by sonication and clarified. Supernatant samples were subjected to SDS-PAGE and Coomassie-stained gels and Western blots prepared. Blots were screened using a sheep anti-LHRH antiserum. The Western blot in Fig. 1A shows the appearance of a band recognised by the antiserum 3 and 4 hours after induction with IPTG. This band corresponds

to a major Coomassie-stained band on the corresponding gel (Fig. 1B) which is at the expected molecular weight for a GST protein containing a single copy of LHRH.

B) Vaccine Production

For large scale preparation of the GST/LHRH protein, shaker flasks were grown and incubated under the conditions outlined above and a cell sonicate prepared as described. The supernatant was purified by glutathione-agarose column chromatography. The GST/LHRH hybrid protein was eluted from the column using 5 mM reduced glutathione in 10 mM Tris HCl pH 8.2. Fig. 2 shows the result of a glutathione column purification. In one step GST/LHRH was eluted from the column in a highly pure state in a yield estimated at 30 µg/ml of the original broth culture.

C) Vaccine Formulation, Immunisation and Testing

Vaccines were adjuvanted with FCA by addition of a third of a volume of conjugate in PBS at one time to the required volume of FCA. The mixture was then emulsified using a Silverson Heavy Duty Laboroatory mixer at maxiumum speed for one minute. This process was performed another two times. The emulsion formed was tested by placing a drop of emulsion on the surface of water. The emulsion was considered stable if the drop remained intact for at least two minutes.

Swiss male mice were used when they reached a mass of > 20gm (approximately 8 weeks of age). They received two doses three weeks apart and were bled two weeks after the second dose. Testes were removed seven weeks after the second dose.

D) Measurement of Anti-LHRH Titres

Dilutions of sera (200ul) in 0.1M NaCl, 10mM sodium phosphate, 0.1% gelatin, 0.1% sodium azide pH 7.38 are added to ^{3}H-LHRH (NEN) (100ul,ca 10000dpm) and 100ul of 0.5% human gamma globulin for 48 hours at 4°C. Cold 18% PEG 6000 (1ml) is added and the tubes are vigorously mixed for 3 minutes and centrifuged at 3000g for 30 mintues. The supernatants are aspirated and the precipitate redissolved and the radioactivity measured. Anti-LHRH titres in sera samples were calculated by plotting the logit of the percentage specific binding versus the log of the serum dilution and interpolating to the dilution giving 30% binding. Titres are expressed as the reciprocal of this serum dilution.

Mouse testes were weighed after mice were killed. Normal mice had testes weights in the range from 0.17-0.32 gm. Male mice were assessed for fertility and sexual activity by caging a test male with 2 mature female mice. After 14-18 days females were killed and the number of foetuses counted. Normal male control mice always produce 6-12 foetuses per female. Treated mice were considered fertile if they produced any foetuses in either feamle. All testes were removed and weighed. Mice having testes weights less than 0.12 gm were invariably infertile while those having testes weights of 0.13-0.16 gm showed reduced fertility (1-6 foetuses in at least 1 female). It is concluded that testes weight of mice when mice had been treated as above, could be used to assess fertility. Any mouse with a testes weight of <0.12gm was deemd to be infertile.

E) Results

Immunocastration of Mice

Three groups of mice were vaccinated in the following protocol:

Group 1: 2 doses each 1mg/ml GST-LHRH in FCA

Group 2: 2 doses each 0.1 mg/ml GST-LHRH in FCA

Group 3: 2 doses each DT/LHRH in FCA

The results are shown in Table 1.

TABLE 1

Antibody to LHRH and Testes Size for Mice Immunised with GST-LHRH Vaccine

Group 1		Group 2		Group 3	
α(LHRH titre	Testis weight	α(LHRH titre	Testis weight	α(LHRH titre	Testis weight
450	0.03 *	170	0.21	1180	0.07 *
350	0.12 *	125	0.16	965	0.06 *
310	0.20	<20	0.18	805	0.23
175	0.23	"	0.19	800	0.04 *
170	0.16	"	0.20	740	0.15
155	0.19	"	0.22	625	0.18
75	0.24	"	0.22	460	0.08 *
<20	0.17	"	0.23	225	0.18
<20	0.18	"	0.24	<20	0.19
<20	0.26			<20	0.22

* Immunocastrated

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within its spirit and scope. The invention also includes

all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

REFERENCES:

1. Schanbacher, 1986

2. U.S. Patent No. 4 608 251
Australian Patent Application No. 22755/88
Australian Patent Application No. 79453/87

3. International Patent Application No. WO 88/09372

4. Australian Patent Application No. 12709/88

5. Smith and Johnson, (1988) Gene 67, 31-40.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A recombinant immunocastration vaccine suitable for use in an animal and capable of stimulating the production of antibodies to LHRH in said animal, said vaccine comprising a polypeptide containing an LHRH amino acid sequence or an analogue thereof, one or more T-cell epitopes and a purification site.

2. The vaccine according to claim 1, wherein the animal is a mammal.

3. The vaccine according to claim 2, wherein the mammal is a livestock animal.

4. The vaccine according to claim 2 wherein the mammal is a companion animal or wild animal held in captivity or in a state of freedom.

5. The vaccine according to claim 2 wherein the mammal is a human.

6. The vaccine according to claim 1, wherein the LHRH amino acid sequence is present more than once in the polypeptide.

7. The vaccine according to claim 1 or 6, wherein the purification site is naturally occurring within the polypeptide molecule.

8. The vaccine according to claim 7, wherein the purification site is the glutathione-binding site on glutathione-S-transferase.

9. The vaccine according to claim 1 or 6, wherein the purification site is a hexa-histidine site.

10. The vaccine according to any one of the preceding claims further comprising a means for self-assembly.

11. The vaccine according to any one of the preceding claims further comprising an adjuvant.

12. The vaccine according to claim 11, wherein the adjuvant does not cause inflammation.

13. The vaccine according to claim 1 wherein the T-cell epitope occurs as a normal component in said polypeptide.

14. A method for immunocastrating an animal, said method comprising administering to said animal an antibody-stimulating effective amount of a polypeptide, said polypeptide comprising an LHRH amino acid sequence or an analogue thereof, one or more T-cell epitopes and a purification site, for a time and under conditions sufficient for said animal to generate LHRH specific antibodies.

15. The method according to claim 14, wherein the animal is a mammal.

16. The method according to claim 15, wherein the mammal is a livestock animal.

17. The method according to claiim 15 wherein the mammal is a companion animal or a wild animal held in captivity or in a state of freedom.

18. The method according to claim 15, wherein the mammal is a human.

19. The method according to claim 14, wherein the

LHRH amino acid sequence is present more than once in the polypeptide.

20. The method according to claim 14 or 19 wherein the purification site is naturally occurring within the polypeptide molecule.

21. The method according to claim 20, wherein the purification site is the glutathione-binding site on glutathione-S-transferase.

22. The method according to claim 14 or 19, wherein the purification site is a hexa-histidine site.

23. The method according to any one of the preceding claims further comprising a means for self-assembly.

24. The method according to claim 14 wherein the T-cell epitope occurs as a normal component in said polypeptide.

25. A vaccine according to claim 1, or a method according to claim 14, substantially as hereinbefore described with reference to the Examples.

DATED this 29th day of April, 1991.

COMMONWEALTH SERUM LABORATORIES LIMITED
By Its Patent Attorneys
DAVIES & COLLISON